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# Parallel Retention of *Pdx2* Genes in Cartilaginous Fish and Coelacanth

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## Abstract

The *Pdx1* or *Ipf1* gene encodes an important homeodomain-containing protein with key roles in pancreas development and function. Mutations in human *PDX1* are implicated in developmental defects and disease of the pancreas. Extensive research, including genome sequencing, has indicated that *Pdx1* is the only member of its gene family in mammals, birds, amphibians, and ray-finned fish, and with the exception of teleost fish, this gene forms part of the ParaHox gene cluster along with *Gsx1* and *Cdx2*. The ParaHox cluster, however, is a remnant of a 4-fold genome duplication; the three other ParaHox paralogues lack a *Pdx*-like gene in all vertebrate genomes examined to date. We have used bacterial artificial chromosome cloning and synteny analysis to show that the ancestor of living jawed vertebrates in fact had more ParaHox genes, including two *Pdx* genes (*Pdx1* and *Pdx2*). Surprisingly, the two *Pdx* genes have been retained in parallel in two quite distantly related lineages, the cartilaginous fish (sharks, skates, and chimeras) and the Indonesian coelacanth, *Latimeria menadoensis*. The *Pdx2* gene has been lost independently in ray-finned fish and in tetrapods.

**Key words:** *Pdx*, *Ipf*, ParaHox, pancreas, coelacanth, Chondrichthyes.

## Introduction

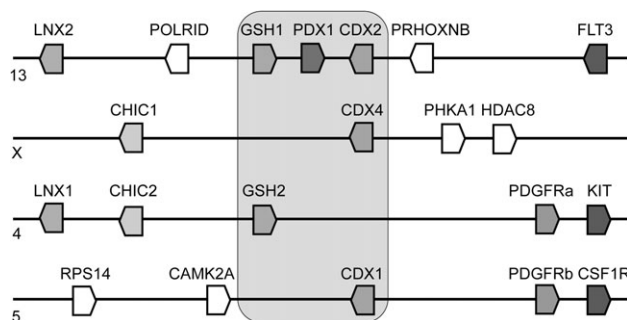
One of the first vertebrate homeobox genes to be described was the *Xenopus laevis* *XlHbox8* gene, expressed in a narrow band of endoderm in the embryo, with pancreatic expression in the adult frog (Wright et al. 1988). This was followed by the identification of the orthologous gene in mouse (*insulin promoter factor 1* or *Ipf1*; Ohlsson et al. 1993) and rat (*islet/duodenum homeobox 1* or *idx1*, also called *somatostatin transactivating factor 1* or *Stf1*; Leonard et al. 1993; Miller et al. 1994). The mouse *Ipf1* gene was mapped to distal chromosome 5 and the human orthologue to 13q12 (Fiedorek and Kay 1995; Stoffel et al. 1995). An orthologous gene was also described in zebrafish, medaka, and *Xenopus tropicalis* (Milewski et al. 1998; Assouline et al. 2002; Illes et al. 2009). The approved gene name according to the gene nomenclature committees for human and mouse is *pancreatic and duodenal homeobox 1* (*Pdx1*), and here, we utilize this nomenclature for the orthologous gene in all vertebrate species.

The vertebrate *Pdx1* gene has a conserved role in the patterning of the midgut in all species examined to date. Mouse *Pdx1* is expressed in the duodenum from around e8.5 and in the dorsal pancreatic bud from e12 (Jonsson et al. 1994). Homozygous *Pdx1* mutants lack pancreatic tissue and parts of the posterior foregut, indicating an essential role for this gene in endodermal patterning in mouse (Jonsson et al. 1994; Offield et al. 1996). Similarly, pancreatic agenesis was reported in a human patient with a homozygous single-nucleotide deletion mutation, which resulted

in formation of a truncated protein (Stoffers et al. 1997). Another patient with pancreatic agenesis was shown to be a compound heterozygote for two different nonsynonymous substitutions within the *Pdx1* homeobox causing reduced protein half-life (Schwitzgebel et al. 2003).

In addition to its role in pancreas development, the *Pdx1* protein is also a glucose-responsive regulator of the insulin gene in the  $\beta$  cells of the adult pancreas and is known to bind to the P1 enhancer 5' of the insulin gene (Ohlsson et al. 1993). As a consequence of this role, mutations in *Pdx1* have also been implicated in type II diabetes mellitus and maturity onset diabetes of the young type IV (Hani et al. 1999; Macfarlane et al. 1999; Cockburn et al. 2004).

The vertebrate *Pdx1* gene was the only identified vertebrate member of the Xlox gene family, also called the *Pdx* gene family, which in turn is within the ANTP class of homeobox-containing genes (Wysocka-Diller et al. 1995; Holland et al. 2007). Members of the Xlox gene family are also present in many invertebrates; indeed, it was studies of the invertebrate amphioxus (*Branchiostoma floridae*) that revealed Xlox to be the central gene in an ancient cluster of three homeobox genes: *Gsx*, Xlox, and *Cdx* (Brooke et al. 1998). Comparison between many animal species indicates that this three-gene ParaHox cluster existed in the ancestor of all chordates and most likely existed in the ancestor of all bilaterian animals (Brooke et al. 1998; Ferrier and Holland 2001; Hui et al. 2009). Synteny analysis in the sea anemone *Nematostella*



**Fig. 1.** Genomic organization of ParaHox genes in the human genome. A cluster of *Gsx*, *Xlox*, and *Cdx* genes is present on chromosome 13. Paralogous genomic regions, generated by genome duplications, are found on chromosomes X, 4, and 5.

*vectensis* further suggests that a ParaHox cluster was present in the genome of the common ancestor of cnidarians and bilaterians, although it did not necessarily include an *Xlox* gene (Hui et al. 2008).

Although amphioxus has only a single ParaHox gene cluster, the ancestral cluster was duplicated 4-fold during early vertebrate evolution, as part of two whole-genome duplications (Coulrier et al. 2000; Pollard and Holland 2000; Ferrier et al. 2005; Putnam et al. 2008). However, there was clearly extensive loss of duplicated genes because humans have only a single complete gene cluster comprising of one *Gsx* (*GSH1*), one *Xlox* (*PDX1*), and one *Cdx* (*CDX2*) on chromosome 13. The remaining duplicates each contain only a single ParaHox gene (*GSH2* or *CDX1* or *CDX4*), but analyses of flanking genes confirm that these four chromosomal regions are indeed descendent from the two genome duplications (fig. 1). In all vertebrates previously examined (human, mouse, chicken, frog, and several species of teleost fish), the complement of ParaHox genes is identical, with two *Gsx* genes (*Gsx1* and *Gsx2*), the one *Xlox* gene (*Pdx1*), and three *Cdx* genes (*Cdx1*, *Cdx2*, and *Cdx4*). The situation is slightly complicated by an additional whole-genome duplication in teleost fish, but basically, the same complement is retained because teleost fish have lost *cdx2* and instead have two copies of *cdx1* (Mulley et al. 2006).

This consistency in gene complement is surprising because in early vertebrate evolution, there may have been up to four of each of these genes following the two rounds of whole-genome duplication. We therefore investigated the ParaHox gene complement of species at informative positions in the phylogeny of vertebrates: the Indonesian coelacanth (*Latimeria menadoensis*) as an outgroup to the tetrapods and three cartilaginous fish—lesser spotted dogfish (*Scyliorhinus canicula*), little skate (*Leucoraja erinacea*), and elephant shark (*Callorhynchus milii*)—as outgroups to bony vertebrates, such as ray-finned fish and tetrapods. We show that in both coelacanth and cartilaginous fish, there is an additional *Pdx* gene which we name *Pdx2*. Phylogenetic and phylogenomic analyses reveal that *Pdx2* was present in the ancestor of all jawed vertebrates but has since been lost independently in both ray-finned fish and tetrapods.

## Materials and Methods

### Isolation of ParaHox Genes in Cartilaginous Fish and Coelacanths

Polymerase chain reaction (PCR) used genomic DNA of Indonesian coelacanth (*L. menadoensis*) and lesser spotted dogfish (*S. canicula*) and nested degenerate primers for the *Xlox* gene family (5′–3′: forward JMXlox1c: GACGACAA-CAAGMGNCANAGRAC; forward nested Xlox2: CAGC TGCTVGAGCTVGAGAA; reverse Xlox3: YTCCTCYTTYTTC-CACCTTCAT; reverse nested XSO2: GCGNCGRTTYTGAAC-CAGAT), the *Gsx* gene family (forward JMGsx1a: ATGY CGMGVTCYTTYWBG; nested forward JMGsx1b: GTNGA YTCNYTVATNWTNARGGA; reverse Gsx3: TTGCCYTCYTYTTGTGCTT; nested reverse GsxSO2: CANCKDCGRTTYT-GRAACCA), and the *Cdx* gene family (5′–3′: forward JmCdx: GGNAARCANMGRACVAARGA; nested forward CdxSO1: CTRGARCTGGARAARGARTT; reverse CdxSO2: NVKNVKRTTYTGRAACCA). Rapid amplification of cDNA ends polymerase chain reaction in *S. canicula* used the SMART RACE cDNA Amplification Kit (Clontech 634914) and pooled embryonic cDNA as template. Full coding sequences of *S. canicula Pdx1* and *Pdx2* are deposited in GenBank under the accession numbers HM142925 and HM142926. Blast searches of the elephant shark *C. milii* partial genome sequence (Venkatesh et al. 2007) were carried out using the Blast server on the Elephant Shark Genome Project homepage (<http://esharkgenome.imcb.a-star.edu.sg/>). In order to analyze the genomic location of *Pdx2*, we screened high coverage bacterial artificial chromosome (BAC) libraries for Indonesian coelacanth (from the Genome Resource Centre, Benaroya Research Institute, Seattle, WA) and little skate *L. erinacea* (from Clemson University Genomics Institute, Clemson, SC) using digoxigenin-labeled *Pdx1* and *Pdx2* homeobox probes. Positive clones were verified by PCR using the above primers prior to sequencing. Coelacanth BAC clone 18814 containing *Pdx2* was sequenced to 9.7× coverage using Sanger sequencing (performed at the Washington University Genome Centre, St Louis, MI); little skate BAC clone 24D8 was sequenced to 45× coverage using Roche 454 GS FLX Titanium technology (performed at the Centre for Genomic Research, University of Liverpool, UK). Genes were predicted using Blast and GenScan and by alignment to known orthologous genes. BAC clone sequences are deposited in GenBank under accession numbers HM134894 and HM134895.

### Phylogenetic Analysis

Orthology and paralogy of *Gsx*, *Pdx*, and *Pdgfr* genes in sequenced BAC clones and related loci in other vertebrates were resolved using phylogenetic analysis. Amino acid sequences were aligned using ClustalX (Larkin et al. 2007) and edited by eye to maximize contiguity of alignable sequence; maximum likelihood phylogenetic trees were constructed with PhyML (Guindon and Gascuel 2003) using the JTT matrix and 1,000 bootstrap replicates. The resulting trees are provided as supplemental information (Supplementary Material online).

Amphioxus Xlox	NKRTRTAYTRGQLELEKEFHFNKYISRPRRIELAAMLNLTERHIKIWFQNRMRMKWKKEQ
Ciona Xlox	.....W.....SR.....D.
Human PDX1	.....A.....L.....V.....V.....E
Mouse Pdx1	.....A.....L.....V.....V.....E
Xenopus Pdx1	.....A.....L.....V.....V.....E
Coelacanth Pdx1	.....A.....L.....V.....V.....E
Zebrafish pdx1	.....A.....L.....V.....LT.S.....E
Fugu pdx1	.....A.....L.....R.....V.....LT.S.....E
Amia calva Pdx1	.....A.....L.....V.....V.....E
Dogfish Pdx1	.....A.....L.....V.....V.....E
Elephant Shark Pdx1	.....A.....L.....V.....V.....E
Coelacanth Pdx2	T.....S.....E
Dogfish Pdx2	G.....T.....E
Elephant Shark Pdx2	S.....V.....II.....E
Skate Pdx2	S.....S.....A.....V.....L.....E

**Fig. 2.** Amino acid alignment of Xlox homeodomains. Only cartilaginous fish and coelacanth have two members of the Xlox gene family—*Pdx1* and *Pdx2*. *Pdx2* sequences shown here were derived from BAC clones (coelacanth and little skate), RACE PCR (dogfish), and genomic sequence (elephant shark).

## Gene Expression Analysis

Expression of *Pdx1* and *Pdx2* in adult dogfish tissues was analyzed using reverse transcription–polymerase chain reaction (RT–PCR). Total RNA was extracted using TRI Reagent (Applied Biosystems Inc., AM9738) in accordance with the supplier's instructions and treated with RNase-free DNaseI (New England Biolabs, M0303) to remove contaminating genomic DNA. Single-stranded cDNA was synthesized using Bioline cDNA Synthesis Kit (BIO-65025) with oligo (dT) priming and PCR carried out using primers ScPdx1f1: AGAGGATCCTACCGTCTCGCATC; ScPdx1r1: CACCGAGTCTCTCGTAGCCGTAG; ScPdx2f1: ACGGATTT CACTGGCTACGACAC; ScPdx2r1: ACCAGATTTTGATGT GCCTCTCG; ScActin1: AGTTGGATGGGTGAGAAAGAC; and ScActinR1: ACGCTCAGTCAGGATCTTCATC.

## Results

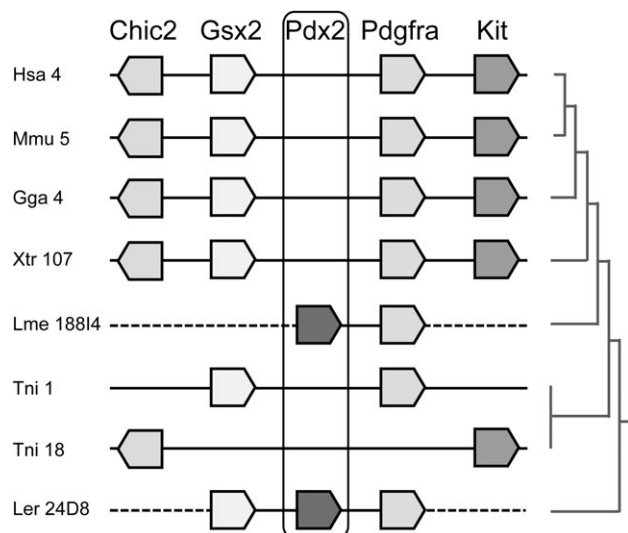
All jawed vertebrates examined previously were found to have an equivalent complement of ParaHox genes, comprising two *Gsx* genes, one *Xlox* gene, and three *Cdx* genes. We asked whether this was the ancestral condition for jawed vertebrates. Key unrepresented informative positions in the evolutionary tree of vertebrates are the coelacanths, representing an early diverging branch within the sarcopterygians, and the sharks, skates, and holocephalians, representing the chondrichthyans. To examine the complement of ParaHox genes in these groups, we first used degenerate PCR on genomic DNA from the Indonesian coelacanth (*L. menadoensis*) and the lesser spotted dogfish (*S. canicula*), plus analysis of partial genome sequence information from a holocephalan, the elephant shark (*C. milii*). The most complete data were derived from the dogfish, which had the expected complement of *Gsx1*, *Gsx2*, *Pdx1*, *Cdx1*, *Cdx2*, and *Cdx4* genes, whereas fragments of all but *Cdx2* were found in the available elephant shark sequence. These analyses also revealed the presence of an extra homeobox sequence assignable to the *Xlox* family, in addition to the expected *Pdx1* gene. The new locus, which we call *Pdx2*, has not been reported in any other vertebrate species examined to date (fig. 2). The full coding sequence of the dogfish *Pdx2* gene was obtained by RACE PCR using pooled embryonic cDNA.

There are three alternative hypotheses that could account for the presence of the additional *Pdx* genes: independent lineage-specific gene duplication in the coelacanth and chondrichthyan lineages, parallel retention of an ancestral vertebrate *Pdx2* gene with independent loss in actinopterygians and tetrapods, or duplication in one lineage (coelacanths or chondrichthyans) with retention of an ancestral gene in the other (chondrichthyans or coelacanths). To resolve between these hypotheses, we first constructed phylogenetic trees to test for orthology. These suggested that the *Pdx2* homeobox sequences of coelacanth and cartilaginous fish form a monophyletic group and are likely to be orthologues rather than independent duplications in each lineage (supplementary information, [Supplementary Material](#) online). However, these initial analyses used only a short sequence length (95 aa) and are not conclusive. To provide a definitive test, we needed to determine the genomic location of the *Pdx2* loci, particularly in relation to their neighboring genes. These could then be compared with the well-studied paralogy groups around the mammalian and teleost ParaHox genes.

Unfortunately, as yet, there is no assembled genome sequence for coelacanth (although a genome project has been approved by the National Human Genome Research Institute) and available sequences from the Elephant Shark Genome Project do not assemble into large contigs, precluding analysis of the *Pdx2* gene neighbors. We therefore screened large insert BAC libraries from coelacanth and little skate (the latter species being closely related to lesser spotted dogfish for which there is no high coverage BAC library).

A *Pdx2*-positive BAC clone was identified in each species and sequenced. First, this allowed us to extend the deduced coding sequence for *Pdx2* from a coelacanth and a chondrichthyan; alignment of these revealed close similarity, further supporting orthology between the two (data not shown). More importantly, the BAC sequencing revealed that the *Pdx2* gene of coelacanth is linked to the *platelet-derived growth factor receptor  $\alpha$*  (*Pdgfra*) gene, and the *Pdx2* gene of little skate is linked to the *Gsx2* homeobox gene and *Pdgfra* gene (fig. 3). In each case, the orthology of the neighbor gene was verified by phylogenetic analysis (supplementary information, [Supplementary Material](#) online). These findings demonstrate that *Pdx2* in both coelacanths and





**FIG. 3.** Genomic neighborhood of the *Gsx2* locus in vertebrates. Coelacanth and little skate have a *Pdx2* gene located between *Gsx2* and *Pdgfra*. Hsa, *Homo sapiens*; Mmu, *Mus musculus*; Gga, *Gallus gallus*; Xtr, *Xenopus tropicalis*; Lme, *Latimeria menadoensis*; Tni, *Tetraodon nigroviridis*; Ler, *Leucoraja erinacea*; numbers refer to chromosome number or genomic scaffold.

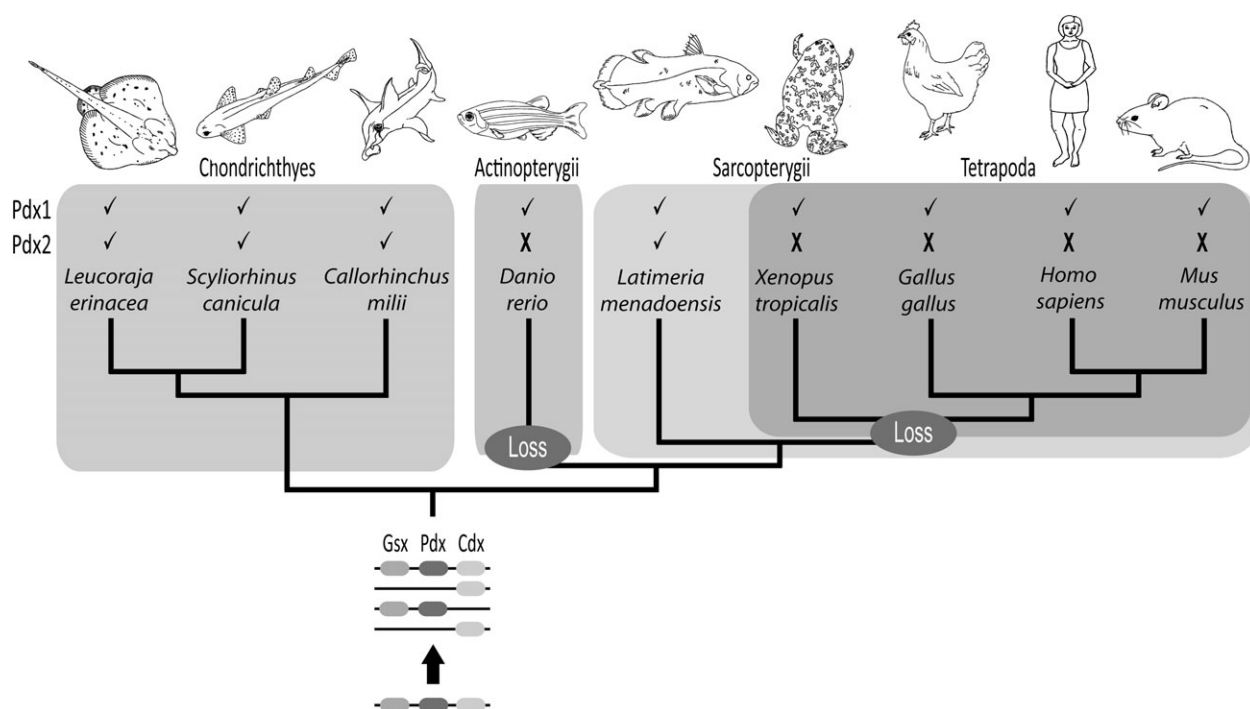
chondrichthyans is located in the ParaHox paralogy region equivalent to that present at human chromosomal location 4q12 (figs. 1 and 3). Indeed, analysis of the orthologous region in teleost fish and tetrapods shows that *Gsx2* is usually located next to *Pdgfra*, with no intervening genes (fig. 3). It is well established that the four ParaHox paralogy regions were generated by two rounds of whole-genome duplication early in vertebrate ancestry. Hence, *Pdx2* must be an ancient

vertebrate gene that was generated during these genome duplication events and was subsequently lost independently in teleosts and tetrapods but retained in parallel in coelacanths and chondrichthyans (fig. 4).

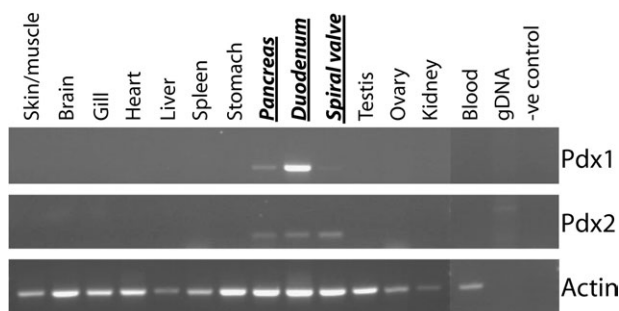
The parallel retention of an ancient homeobox gene in two widely divergent evolutionary lineages raises intriguing questions about the roles of the *Pdx1* and *Pdx2* genes. In order to shed light on this subject, we carried out RT-PCR using RNA extracted from adult dogfish tissues. This revealed that the *Pdx1* gene is expressed most strongly in the pancreas and associated duodenum, with weak expression in the spiral valve. The *Pdx2* gene shows a similar expression profile and therefore overlaps with *Pdx1*, although *Pdx2* is expressed more highly in the spiral valve (fig. 5). Analyses of gene expression in coelacanths are not currently possible because of their rarity and protection under Appendix I of the Convention on International Trade in Endangered Species.

## Discussion

Homeobox genes have provided important insights into vertebrate genome evolution and gene family diversification. We have investigated the pattern of evolution of the developmentally and medically important ParaHox gene family in vertebrates using species chosen for their phylogenetic positions. One question asked was why is there an apparently stable complement of ParaHox genes in jawed vertebrates, comprising two *Gsx* genes, one *Xlox* gene (*Pdx1*), and three *Cdx* genes? Here, we show that in fact, this complement is not stable nor is it retained in all vertebrate lineages. We have identified a previously



**FIG. 4.** Evolutionary history of ParaHox genes in vertebrates. The vertebrate ancestor had a *Pdx2* gene linked to *Gsx2* in a ParaHox paralogon; this gene has been lost independently in ray-finned fish and tetrapods and retained in parallel in cartilaginous fish and coelacanths.



**Fig. 5.** Expression of *Pdx1* and *Pdx2* in adult dogfish tissues. RT–PCR for both *Pdx* genes in a number of different tissues reveals that *Pdx1* and *Pdx2* expression is restricted to the pancreas and duodenum (including the spiral valve).

unknown member of the *Xlox* gene family (*Pdx2*) and, by analyses of its genomic context, discovered that this gene was present in the ancestor of all living jawed vertebrates. Intriguingly, however, *Pdx2* has been retained during vertebrate evolution only in cartilaginous fish and coelacanths (fig. 4), two quite distant lineages, and has been lost independently in ray-finned fish and tetrapods.

The retention of both *Pdx1* and *Pdx2* genes in any living vertebrate indicates that the two genes cannot have completely equivalent roles; otherwise, one gene would have been lost by mutation and degeneration in the 450–550 My since their origin. The earliest stage of this functional distinction between *Pdx1* and *Pdx2* is likely to have occurred at the gene regulatory level, possibly involving loss of ancestral (preduplication) roles in each of the daughter genes in a complementary manner (subfunctionalization; Lynch and Force 2000). It may have taken tens of millions of years for these regulatory mutations to accumulate sufficiently for gene expression divergence, judging by evidence from other gene families, such as neurogenin (Furlong and Graham 2005) and snail (Locascio et al. 2002). For example, mammals and teleost fish express *neurogenin 1* (*Ngn1*) in the ophthalmic trigeminal placode where birds use *neurogenin 2* (*Ngn2*); this implies that *Ngn1* and *Ngn2* were both expressed in this placode prior to the divergence of birds and mammals, with their differential expression evolving long after the actual duplication event (Furlong and Graham 2005). If a similar process took place with vertebrate *Pdx1* and *Pdx2*, then for a substantial period of time after their origin they will have had identical roles, followed by stepwise subfunctionalization over millions of years and possibly addition of new functions. However, even though there must be functional differences between *Pdx1* and *Pdx2* in living vertebrates, our analyses of gene expression in dogfish have not thus far revealed any clear distinction between the two genes, with both being expressed in pancreas, duodenum, and the spiral valve. Because the subfunctionalizing mutations will have occurred independently in chondrichthyans and in coelacanths, we should not assume that the two genes are performing exactly the same role in these two evolutionary lineages.

It is clear that the *Pdx1* and *Pdx2* genes were generated in the genome duplication events that occurred during early

vertebrate evolution and that both were retained when the jawed vertebrate lineage split into the Chondrichthyes (cartilaginous fish) and Osteichthyes (bony vertebrates). In the lineage leading to humans, the Osteichthyes, the *Pdx2* gene was retained until the divergence of Actinopterygii and the Sarcopterygii, and in latter lineage, it was still present at the divergence of the coelacanth and tetrapod lineages. The gene was then lost independently within the Actinopterygii and in the tetrapods, before the evolutionary radiation of the living tetrapod lineage. Lungfish are the only group of animals likely to have diverged in this period, being the probable sister group to tetrapods (Takezaki et al. 2003). In an attempt to refine the timing of *Pdx2* gene loss in sarcopterygians, we used PCR to search for *Pdx* genes in the Australian lungfish (*Neoceratodus forsteri*). We identified only the *Pdx1* gene, tentatively suggesting that *Pdx2* may have been lost in the ancestor of lungfish and tetrapods (data not shown). As to why *Pdx2* was lost twice in vertebrate evolution, in tetrapods and in teleosts, it is key to recall the process of duplicate gene divergence discussed above. If functional divergence occurred over a period of tens of millions of years, then the two genes could still have had substantially overlapping roles for the entire time between the gene duplication event and the divergence of Actinopterygii and the Sarcopterygii. This period may have been less than 100 My, if we consider the genome duplication date to be 450–550 Ma and the Actinopterygii/Sarcopterygii divergence as 425–476 Ma (Blair and Hedges 2005). After that phylogenetic node, we deduce that disabling mutations occurred in *Pdx2* independently in a teleost ancestor and a tetrapod ancestor and were viable because of redundancy with *Pdx1*. We assume that it would have been possible to lose either one of the genes and still maintain full function, and thus, it is coincidence that both teleosts and tetrapods both lost *Pdx2* rather than *Pdx1*. An alternative explanation is that the full function could only be fulfilled by *Pdx1*, perhaps because of some feature of its genomic location, but we find no evidence to support this suggestion because both *Pdx* genes are linked to *Gsx* genes, whereas teleost fish genomes demonstrate that *Pdx1* can function without a neighboring *Cdx2* gene. Either way, the independent loss of *Pdx2* in teleosts and tetrapods, and its parallel retention in chondrichthyans and coelacanths, presents a particularly unusual pathway of molecular evolution for vertebrate homeobox genes.

## Supplementary Material

Supplementary materials are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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